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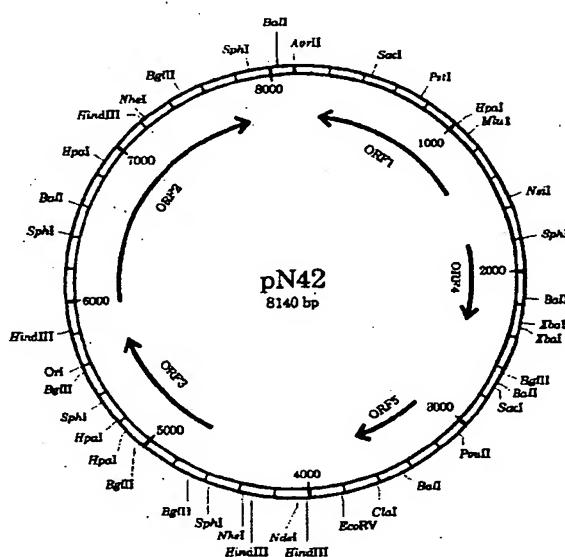
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(54) Plasmid derived from Lactobacillus delbrueckii sp.

(57) The present invention concerns a plasmid derived from Lactobacillus delbrueckii sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof; the recombinant vector comprising the said plasmid, at least one DNA sequence capable of replication into E. coli and/or Lc. lactis and at least one marker.

The present invention concerns also the microorganism transformed by the said plasmid and/or by the said recombinant vector.

FIG.1



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Field of the invention

The present invention concerns a new plasmid derived from *Lactobacillus delbrueckii* sp., a recombinant vector comprising said plasmid, the microorganism transformed by said plasmid and/or vector and the use of the plasmid and/or the vector for the transformation of microorganisms.

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Background of the Invention and state of the art

A successful biological transformation of an organism must satisfy the following three criteria:

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1. Transforming DNA must enter the organism by physical or chemical means such as electrotransformation, treatment with inorganic ions, protoplast fusion, etc.

2. Transformants must be selected with the help of one or more markers from the non transformed cells in the population for instance by antibiotic resistance genes linked to the transforming DNA. This is best satisfied by either the isolation of a resistance gene against an antibiotic from the target host in question, or by the engineering of a known resistance gene with expression sequences (promoter and terminator) compatible with the target host.

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3. Transforming DNA must be replicated (either autonomously or as part of the host genome). This is best satisfied by the isolation of replicating plasmids from the host to be transformed and to subsequently construct vectors able to replicate in a microorganism such as *Escherichia coli* (*E. coli*) or *Lactococcus lactis* (*Lc. lactis*) and in a specific target organism such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*).

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The international patent application W092/14825 describes a plasmid pBULL having a length of about 7.9 kb and its derivative isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* M-878 strain.

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The restriction map of this plasmid is characterized by the absence of restriction sites for BamHI, EcoRI, KpnI and PstI enzymes.

This plasmid is used as a vector for breeding various microorganisms such as lactic acid bacteria and the derivative of this plasmid is used as a shuttle vector (lactic acid bacterium - *Escherichia coli*). X

Other shuttle vectors are described in the documents Canadian Journal of Microbiology (vol. 38 (1992) pp 69-74), ACTA MICROBIOLOGICA BULGARICA (vol. 27 (1991) 99 3-8) and in the Japanese Patent Application JP-A-4.218.381.

Aims of the invention

The present invention aims to provide a new plasmid derived from *Lactobacillus delbrueckii* sp. which can be used to transform specific microorganisms specially *Lactobacillus bulgaricus*.

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Another aim of the invention is to obtain a recombinant vector comprising the said plasmid and which can replicate in *E. coli* and *Lc. lactis* and transform specific microorganisms, specially *Lactobacillus bulgaricus*.

Disclosure of the invention

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The present invention concerns a new plasmid derived from *Lactobacillus delbrueckii* sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof.

Preferably said portion is a sufficient amount of the restriction map of the Figure 1, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in *Lactobacillus bulgaricus*. (S)

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The plasmid according to the invention comprises at least the DNA sequence SEQ ID N° 1 and/or its complementary strand, or portion(s) thereof. (S)

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Preferably, said portion is a sufficient amount of the DNA sequence SEQ ID N° 1 and/or its complementary strand so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in *Lactobacillus bulgaricus*. (S)

Furthermore, the present invention concerns a recombinant vector comprising the plasmid according to the invention, at least one DNA sequence capable of replication in *E. coli* and/or *Lc. lactis* and at least one marker.

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The DNA sequence capable of replication in *E. coli* and/or *Lc. lactis* is constituted for instance by a specific plasmid, such as pDP193, which allows the recombinant vector to be freely cultured in either *E. coli* or *Lc. lactis* for molecular manipulations.

The marker comprised in the recombinant vector according to the invention, is a DNA fragment used as a reference for analytical purposes (i.e. a gene with known phenotype and mapped position) and/or a foreign

DNA fragment which is expressed in the microorganism transformed by the vector according to the invention.

This DNA fragment may be used also for the transformation of microorganisms in order to obtain for instance:

- resistant strains to phages,
- ropy strains (improved texturing properties),
- probiotic strains,
- strains producing new or improved enzymes (lipases, deshydrogenases,...), aroma or flavor compounds.....

The present invention concerns also the microorganism, preferably *Lactobacillus bulgaricus*, transformed by the plasmid and/or by the recombinant vector according to the invention.

Finally, the present invention concerns the use of the plasmid and/or the vector according to the invention for the transformation of microorganisms.

#### Brief description of the drawings

- 15 The Figure 1 represents the restriction map of the *Lactobacillus delbrueckii* sp. plasmid pN42 according to the invention.
- The Figure 2 represents the construction of the plasmid pN42-Sub CB from the pJDC9 plasmid and pN42 plasmid.
- 20 The Figure 3 represents the construction of pN42-Sub CE from the pJDC9 plasmid and pN42 plasmid.
- The Figure 4 represents the construction of pN42-Sub W and pN42-Sub X from the pUC19 plasmid and pN42 plasmid.
- The Figure 5 represents the construction of chloramphenicol transacetylase gene of pDP352.
- The Figure 6 represents the construction of the pDP193 plasmid.
- 25 The Figure 7 represents the construction of pDP359 plasmid.

#### Description of a preferred embodiment of the invention

The construction of pDP359, a *E. coli/Lc. lactis-L. delbrueckii* sp. shuttle vector according to the invention is characterized by the following features.

Firstly the incorporation of pDP193 allows the plasmid to be freely cultured in either *E. coli* or *Lc. lactis* for molecular manipulation, such as the addition of genes to be expressed in *L. bulgaricus*. Secondly the inclusion of a bona fide *L. delbrueckii* sp. plasmid in its entirety ensures that pDP359 contains all the sequences required for the replication of pN42 and hence must replicate in *L. bulgaricus* in the same fashion as pN42 in its host N42. Thirdly the inclusion of the chloramphenicol resistance gene engineered in pDP352 ensures a means to select for transformants in *L. bulgaricus*.

Analysis of over fifty *L. delbrueckii* sp. strains from the Nestle culture collection identified one, N42, that contains an extra-chromosomal replication plasmid. This is designated pN42 (its restriction map is shown in the figure 1) and chosen for analysis as it must contain all of the plasmid encoded TRANS and CIS elements necessary for its replication in *L. bulgaricus*. The integrity of N42 as a *L. delbrueckii* sp. is ascertained by API tests and molecular characterization of hybridization with the *L. delbrueckii* specific probe (Delley M., Mollet B., and Hottinger H., 1990, DNA probe for *Lactobacillus delbrueckii*, Appl. Environ. Microbiol., 56: 1967-1970).

pN42 plasmid DNA is isolated by cesium chloride ethidium bromide buoyant density gradients for restriction mapping and sub cloning. Plasmid pN42 is cloned in its entirety into the *E. coli* vector pJDC9 (J.-D. Chen and D.A. Morrisson 1987, Cloning of *Streptococcus pneumoniae* DNA Fragments in *Escherichia coli* Requires Vector Protected by Strong Transcriptional Terminators, Gene 55, 179-187) at several identified unique restriction sites PstI (pN42-Sub CB), AvrII (pN42-Sub CE) or into the pUC/pK plasmids for DNA sequence analysis.

pN42 plasmid DNA is digested with the restriction enzyme PstI, mixed with PstI digested and dephosphorylated pJDC9 vector, ligated and transformed into *E. coli*. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pN42-Sub CB (figure 2).

pN42 plasmid DNA is digested with the restriction enzyme AvrII, mixed with XbaI digested and dephosphorylated pJDC9 vector, ligated and transformed into *E. coli*. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pN42-Sub CE (figure 3).

Plasmid pN42-Sub CB is digested with the restriction enzymes EcoRV and PstI, the DNA fragments separated on an agarose gel and the 3.1 kb and 5.1 kb fragments purified. These two fragments are mixed with PstI and SmaI digested and dephosphorylated pUC19 vector, ligated and transformed into *E. coli*. Colonies are analyzed by restriction enzyme digestions and the positive clones designated pN42-Sub W and pN42-Sub X (for the 5.1 kb and 3.1 kb fragments respectively) (figure 4).

The complete DNA sequence of pN42 is determined from subclones from synthetic oligonucleotide primers on both strands by the dideoxy chain termination reactions using the <sup>35</sup>S sequencing® kit of Pharmacia and <sup>35</sup>SdATP. pN42 consists of a circular double stranded plasmid of 8140 base pairs with at least five open reading frames (designated ORF1 to ORF5) of 50 amino acids or more as identified by the computer program "Frames" from the GCG suite (Computer software is from Genetics Computer Group Inc. (GCG), Devereux J., Haeberli P. and Smithies O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387-395). The GCG program "Repeat" identified a three times twenty-one base pair direct repeat which is the potential origin of replication. The restriction map of pN42 is shown in Figure 1 and the complete DNA sequence in sequence listing-1 (SEQ-ID-N<sup>o</sup>.1).

The DNA sequence analysis of pN42 allows the definition of structural features that may be important for the replication of the plasmid in *L. delbrueckii* sp. and the construction of shuttle vectors that include all these features intact (the introduction of genes may be obtained by cloning pN42 at the following restriction sites Avr II, NsiI, SphI, Nb plasmid DNA isolated from *Lactobacillus delbrueckii* sp. digested at only one of the five SphI sites I.E. at bp 7882).

This ensures that the said shuttle vector must replicate when transformed into *L. bulgaricus*. It is judged probable that antibiotic resistance conferred by a defined resistance gene may be transferred to any other organism if it contains the appropriate translation/transcriptional control signals. Therefore the defined gram positive chloramphenicol resistance gene (chloramphenicol acetyltransferase, CAT originally from *Staphylococcus aureus*) is been taken from the broad host range plasmid pNZ12 (W.M. de Vos, 1987, Gene Cloning and Expression in Lactic Streptococci, FEMS Microbiol. Reviews, 46, 281-295) and used to engineer the bona fide *L. bulgaricus* promoter from the lacS-Z operon (P. Leong-Morgenthaler, M.C. Zwahlen and H. Hottinger, 1991, Lactose Metabolism in *Lactobacillus bulgaricus*: Analysis of the Primary Structure and Expression of the Genes Involved, J. Bacteriol., 173, 1951-1957). This is followed with a gram positive stem-loop terminator from the lactose-galactose operon of *Lc. lactis* strain NCDO2054. The complete construction is shown in Figure 5.

The plasmid pKN19 is the *E. coli* cloning vector pK 19 (R.D. Pridmore, 1987, New and Versatile Cloning Vectors with Kanamycin-Resistance, Gene, 56, 309-312) where the unique BspHI restriction site in a non essential region is destroyed by restriction enzyme digestion and the four base overhang repaired with Klenow enzyme and the four nucleotides according to Maniatis et al. (T. Maniatis, E.F. Fritch and J. Sambrook, Molecular cloning a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982). The chloramphenicol resistance gene from pNZ12 is extracted by PCR amplification (Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B., and Ehrlich H.A., 1988, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239: 487-491; Saiki R.K., Scharf S., Faloona F., Mullis K.B., Horn G.T., Ehrlich H.A. and Arnheim N., 1985, Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230: 1350-1354) using the mutagenic primers A (5'-AGGAGGATCTCATGAACTTAATAAAATTG) that introduced a BspHI restriction site overlapping the ATG initiation codon of the CAT gene, plus primer B (5'-TACAGTATCGATTATCTCATATTATA) that introduces a Cial restriction site 9 bp down stream of the CAT gene. The PCR amplification is performed on 50 ng of BgIII digested pNZ12 DNA with 0.3 μM each of oligonucleotides C plus D, 200 μM of the four nucleotides and PCR cycling at 94°C for 0.5 minutes, 50°C for 0.5 minutes, 72°C for 0.5 minutes for a total of 30 cycles.

The product is digested with the restriction enzymes Cial plus BamHI and the 660 bp fragment purified from an agarose gel and cloned into the *E. coli* vector pBS KS+® (Stratagene Corp.) also digested with Cial, BamHI and dephosphorylated. The ligated fragments are transformed into *E. coli* and plated onto LB plates supplemented with ampicillin, 5-bromo-4-chloro-3-indolyl-(3-D-galactopyranoside) (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). Clones are screened by restriction enzyme digestions, a positive clone chosen and designated clone A; both chloramphenicol and ampicillin resistant. Clone A is digested with restriction enzymes MfeI, StuI and dephosphorylated. This fragment is replaced by the equivalent CAT MfeI-StuI fragment from pNZ12. This is to eliminate any PCR induced mutations within the CAT gene, giving Clone B. (This step is not shown in Figure 5).

Clone B is digested with the restriction enzymes BamHI plus Cial and the 660 bp fragment purified from an agarose gel. pKN19/galT-term is pKN19 containing the *Lc. lactis* NCDO2054 lactose-galactose operon terminator as an SpeI-SacI restriction fragment, with its internal BspHI restriction site destroyed as described above. pKN19/galT-term is digested with the restriction enzymes SphI plus SacI (both sites natural to the fragment) and the 190 bp fragment purified from an agarose gel. These two fragments are mixed together with the vector pKN19 digested with the restriction enzymes SacI, BamHI plus dephosphorylated, ligated together and transformed into *E. coli*. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated clone C.

5 The published *L. bulgaricus* lacS promoter is used to design two mutagenic oligonucleotides, C (5'-ATTG-GAAGAATTCAACGCTTTCATTC) which introduces an EcoRI restriction site 240 bp upstream of the ATG initiation codon and oligonucleotide D (5'-GGTGGTGACGAAGACGATA) which primes 110 bp downstream of the ATG of the lacS gene which naturally contains a BspHI restriction site overlapping the start codon. The PCR amplification is performed on 100 ng of genomic *L. delbrueckii* sp. DNA with 0.3  $\mu$ M each of oligonucleotides C plus D, 200  $\mu$ M of the four nucleotides and PCR cycling at 94°C for 0.5 minutes, 50°C for 0.5 minutes, 72°C for 0.5 minutes and a total of 30 cycles. The PCR product is digested with the restriction enzymes EcoRI plus BspHI and the 250 bp fragment purified from an agarose gel. Clone D is digested with the restriction enzymes BspHI plus SacI and the 780 bp fragment purified from an agarose gel. These two fragments are ligated together into EcoRI, SacI plus dephosphorylated pKN19 vector, transformed into *E. coli*, and plated onto LB plates supplemented with kanamycin. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated pDP352 the complete DNA sequence of which is given in sequence listing 2 (SEQ ID No. 2).

10 15 The chloramphenicol resistance gene constructed in pDP352 is transcribed from a bona fide *L. bulgaricus* promoter that is constitutively expressed in this host. This includes the natural promoter elements of -35, -10 regions and the ribosome binding site at exactly the same relative position to the ATG of the chloramphenicol resistance gene as to the original ATG of the lacS gene. This ensures that the chloramphenicol resistance gene will be correctly transcribed and translation initiated at the correct position and that the resistance gene will work.

20 25 The *E. coli*-*Lc. lactis* shuttle vector pDP193 is constructed from the *E. coli* vector pUC18 (R.D. Pridmore, 1987, New and Versatile Cloning Vectors with Kanamycin-Resistance, Gene, 56, 309-312) plus the plasmid pVA749 (F.L. Macrina, J.A. Tobian, K.R. Jones and R.P. Evans, Molecular cloning in the Streptococci, in A. Hallander, R. DeMoss, S. Kaplan, S. Konisky, D. Savage and R. Wolfe (Eds.), Genetic engineering of microorganisms for chemicals, Plenum, New York, 1982, pp. 195-210). pVA749 is extracted from the chimeric plasmid pVA838 (F.L. Macrina, J.A. Tobian, K.R. Jones, R.P. Evans and D.B. Clewell, 1982, A Cloning Vector able to Replicate in *Escherichia coli* and *Streptococcus sanguis*, Gene, 19, 345-353) as a HindIII restriction fragment and cloned into the HindIII site of pUC18. The second HindIII site opposite to the pUC cloning array is removed by Klenow enzyme end repair. pVA749 itself consists of a gram positive plasmid origin of replication from *Streptococcus faecalis* (capable of replication in *Lc. lactis*) and the erythromycin resistance gene from pAMβ1. The construction of pDP193 is depicted in Figure 6.

30 35 Plasmid pVA838 is digested with the restriction enzyme HindIII, the fragments separated on an agarose gel and the 5.2 kb pVA749 fragment purified. Vector pUC18 is digested with the restriction enzyme HindIII, dephosphorylated, mixed with the pVA749 fragment, ligated and transformed into *E. coli*. Colonies are analyzed by restriction enzyme digestions and a positive clone designated Clone D. Clone D is digested with the restriction enzyme HindIII in the presence of 50  $\mu$ g/ml ethidium bromide (M. Osterlund, H. Luthman, S.V. Nilsson and G. Magnusson (1982), Ethidium-bromide-inhibited restriction endonucleases cleave one strand of circular DNA, Gene 20, 121-125), the fragments separated on an agarose gel and the linear 7.9 kb fragment purified. The four base overhang generated by HindIII in the linear Clone D is filled in with Klenow enzyme in the presence of four nucleotides according to Maniatis et al. (T. Maniatis, E.F. Fritch and J. Sambrook, Molecular cloning a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), ligated and transformed into *E. coli*. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pDP193.

40 45 Plasmid pDP193 is digested with the restriction enzymes SacI plus EcoRI and dephosphorylated. pDP352 is digested with the restriction enzymes SacI plus EcoRI and the 1100 bp CAT gene purified from an agarose gel. These two are mixed together, ligated and electrotransformed into the *Lc. lactis* plasmid free strain LM0230. Positive colonies are identified as erythromycin plus chloramphenicol resistant and confirmed by restriction enzyme digestions. A positive clone is chosen and designated pDP193-CAT 352.

50 pDP193-CAT 352 is digested with the restriction enzymes SseI plus BamHI and dephosphorylated. Plasmid pN42-Sub CE is digested with the restriction enzymes SseI plus BamHI (both sites from the linker) and the 9.3 kb fragment purified from an agarose gel. These two fragments are mixed, ligated and electrotransformed into *Lc. lactis* strain LM0230. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated pDP359 as shown in figure 7.

55 The vector pDP359 satisfies the requirements for a shuttle vector for *L. bulgaricus* that must work in this host. It includes a complete bona fide replicating plasmid isolated and characterized from *L. delbrueckii* sp. plus a chloramphenicol resistance gene that is transcribed from a native *L. bulgaricus* promoter. These considerations ensure that the said plasmid pDP359 which replicate when introduced into *L. bulgaricus*.

## SEQUENCES LIST

## 5 Information for sequence ID No 1.

## (i) Sequence characteristics:

10 (A) Length: 8140 base pairs  
(B) Type: Nucleic acid  
(C) Strandedness: Double  
(D) Topology: Circular

## 15 (ii) Molecule type: DNA (plasmid)

## (xi) Feature:

(vi) Original source: Lactobacillus bulgaricus Strain N2.  
(A) Name/Key: Plasmid pN42  
(B) Location: 1..8140

## 20 (XI) feature:

(A) Name/Key: Origin of replication.  
(B) Location: 5694..5758.

## 25 (XI) feature:

(A) Name/Key: ORF1.  
(B) Location: 1344..169.

## (XI) feature:

(A) Name/Key: ORF2.  
(B) Location: 5965..7806.

## (XI) feature:

(A) Name/Key: ORF3.  
(B) Location: 4718..5668.

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## (XI) feature:

5 (A) Name/Key: ORF4.  
 (B) Location: 3116..3637.

## (XI) feature:

10 (A) Name/Key: ORF5.  
 (B) Location: 1779..2360.

*JN42*

15 CCTAGGCTTG AAATTGACGC ATAGGCGCAA AGGGAGCGGG CGACAGGGGG TAAAGCACGA 60  
 TAAATTCGTT TTTTACAAGAC GTTCAGTCGA TGTTGTCATA TTTGTACTCC CGTTTTAGG 120  
 GCTGTTTAA AAGTATTTT AGCGGCGATT TGTTAATTAT AGCCCCTATA CAAACATCTT 180  
 20 TTGAAAAAG CCTTTTTCT GTTCTTCAA CAAATCTAAC TTACGTTGAT GAAGAGCGAT 240  
 AGTGTCACT AGCTGTTTA AAAATGAGCC TATTTTTTT TGTTCTTCCT GACTAGGTTT 300  
 ATAGATTTA AATGATGAAA ATTTAGAAAT CCAATGACGT TCATGACTTT GAGGTACATA 360  
 25 TTTTATATTC TTCAATGTAT TAAACATAAA ATAGAAATTG TCAGAATTAT CATTCAAAC 420  
 AAGTAATTTC ATTGCGGAGC TCTTAATTAA AAAAGGGAAA TCTACATAAT GAGAGTCAGT 480  
 TGTAATCA TCAAATATAA CAACTGGATT TTCTACGGTA GCATTTTAA TCCCGCTAAT 540  
 30 TTCATCTGTA TAGCCCAATA AGAAAATCTT GCCTGCTGTT AAAACAGGGG TATTAAAATT 600  
 GTCATCGTAC TCTGTAGATT TGACAATATA TTTTGTGGT TGCTCATAGT TAAATACCTC 660  
 CCCCAACTTA CACTGCTCCC ATTGTCACT AAATCCTTCA AACCGAATAG CTGGATACCC 720  
 35 GCTCTTATAA GCGAACATTT TCTGCAGTAA AGCGTTTTT AAGCATTAA GTTGCTGTTT 780  
 CTTTCTCTCA TGTAAAGTGA TTGCAGTATC CAATTCAAGAG AAGAAGTTAG CAATTCTTC 840  
 TTGTTCAAGAC GTAGTTGGAA ACGAACAGA CTGATTCCG ACAATATCCG AGTTCAAATT 900  
 AACCTGACTT CCCGGCTGAC CATATTGTT CCAATATGGT TTGAACATAA GAAGCCATTG 960  
 40 AACATATAAT TCCTTATAA ATGTTGGGTT GAGAAATATT AAGAATCCAT CGTGAACCTCC 1020  
 TGTGTTAACG TAATTGATCA CTGGACTACC CACAGTAGCA GCAATACTTA ACAATAATG 1080  
 TGGTTCTGTG ATAACACCGCG TTTAGATTG ACCAGCTTT GAAATGTGTT GCGATAAGTG 1140  
 ATGAATGCGT CCTTTTTGTT CAGTGACATC GGATATTCTT AGCCATCCAA CATTGAAATT 1200  
 45 ATCATCGAAC CATTGGGGT TAGAAATAGG TCTTGGACTC GCTCCACGTA CGATTTCCGC 1260  
 TTTGTTTTT AACTTACACT GCTCCAAGG ATCAGCGAAA CCTTTAAATC TTAATTGCGG 1320  
 ATATTTAGCT TGTGTATCAT TCATTATTTT TCCTCCGGTT TAATGTCTAA GGCCATTAA 1380  
 TCAAATTAAA AATCAGCAAA ACCTATTTG TGTCTGGTGG AACCAACAAG CGGCTAGAAA 1440

50

ATATGCTGCC AAACACCCCTA AAGAACAAAA TATTGATAAC GAGCATACTT GGCAATTAAAC 1500  
 5 GCCGTATAAG CTCATTTAAG CCGTTTTAAG TGTTATATGC ATAATTATAT TAAAAGTGCT 1560  
 TTAAAATCGC TTAGAAGCAA GAATAGGCAG CTTGAGTGGC TGAATTGGCG ATGACTGAAC 1620  
 TAAGGACTAG GCCAAGAAAAC TTTGCACAG TCAACAATTG CCCGGACTAA TTCGGACTTT 1680  
 10 TTCTTTCTGG TCAGGTCTCC TAATGGTCAG TAAGGTAGC CGCTTCAGCG GTCAATCGT 1740  
 TATAATAATA ATCAAGATTG ACAAGAGGAG GGCTGACAAT GGCAAATAGC GCTGGCATGC 1800  
 TGTCAGTAGG TCAAATAGCT AAAATGCTGA AGACCAACAG ACAGAACATT TACAACGTGC 1860  
 15 TTAAAGCTGA GCATATTAAG CCTGACGGCT TCAATGACAA GCACTATTCA CTTTACAGCC 1920  
 CGGAAACAAT TCAAGAGATC AAGGCCGCTC TGTCTAAGAA GGCAACGCTG AGAAGTAAGA 1980  
 AGGTAGTAGG AAAAGAGCAG GCTGAAGAGA TAGCTGACTT GAAGAACATCAG CTGTCAGAAC 2040  
 20 AGCAGAGATT GACAACCTGG CTACAGTCTC AGCTGGTTCA ACTTCAAGTA GAGGCTGACA 2100  
 AGCTCAGGAG TCAGAACAGC CAGTTACAGC TAGACAATGC AAAGACTCAG CTCCTTATTG 2160  
 GCCAGGTTGA CCAGGAGAAG ACAACACTGA AGGCCGAGAA TGACCGACTG AGCGCTGAAA 2220  
 25 ATAACAAACT AGGACAATTG ACCGATAAGG TGCTGAAGGA CGCTCAGAGA GCAGAACAGG 2280  
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 CTAGAACATCAC CAGGAATTAT TAAGAGTGGT ATAGCCGTTA TCTGACTTTG TGAAATTCC 2400  
 30 TATTGGCTCT GTCAGATCAA GCGATTTAA ACCTATACGA GTTGTGAAT CCTAGTTAC 2460  
 GGAATTGGGC GATAAGGAAG CCCGTCATTG CAAGGATAGA AGGTTAGTTC CAATAAGACA 2520  
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 35 AGACACTTGC CAGCATTGAC TGTAGCGGCT TTACAATGAC ACTAGATCTA CACTATAATT 2640  
 ACAGCGAAA GAGAAAGGCT GAGCGGTCTC CTAATGGACA ACTACAACCTG GCCAGCCGG 2700  
 CAACTTGAG AGCCGTTAAA GAGCTCTCTC AGCATGGTTA GAGTATAGAA AGAGTGCTGA 2760  
 40 ACATGGACTT TAAAAAAGGG CTGAAGGGCT TGCAAGATCA GCAGACCCGG CTTGAAGCTA 2820  
 AACAGGAAGT ACTGTTAGAC ATCATGGCTG AGTTCTGGCC TAAAGTAGCT AAAGAACGGCA 2880  
 ATGACGTTGC TGAAGCGGTC AAGGTAGAAG ACCTGGCTGA ATGGTTCGCT AAGAACAGCC 2940  
 45 CGAAAATGT TATTGCGTG TCAGCAAGAC AGAAGACGGC TATGACCTGG CTTTGAAACC 3000  
 ACAACAGCCT TCAAGAGAAT TGTTATGGTA CGATGATCTT TATTGGCGGC TGGGTAAAAC 3060  
 AGCTGACCAA CTCAAAACGT AAATCTAAGG TCAAGACGCT AGAGGAAATT ATCTAATGGC 3120  
 50

5           GGTTTACAAA GAATGGACTG ATTCAGATCA TTTAGAGTTA GTCAAAAATT GGAAATTACA 3180  
 CCGGCTGA  ACT AACGTTGAGA TAGCTCAAAG AATAGGCATT GCTGAGAAGA CTTTGATCGT 3240  
 ATGGTTGAAG AAGTCTCCTA AGCTGAAGAA GGCCATTAGA GGCGGCAAGG ATATTGCCAG 3300  
 10          GGCTAGGGCT GAGAATGCAC TGTATGAGCT TGCTCTTAAT GGCGATAGGC AAGCCCTTT 3360  
 CTTTGGCTC AAAAACAACT ACAGAGAACG CTACTCAGAC AAGCCGTTAA GCCCGGCTGA 3420  
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 TCAGCTGAAG GCCATTAAGG AAGACCAGGG AGACCAAGCA ACGCAATTAA ACAACCTGTT 3540  
 15          AGACAGTCTG AAGGAAGCCG TGTTAGATGA GGGATTAGC CCCGATAACA TCGTTCTAC 3600  
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 GTAAACACAA GATAGCGGAA AATCTTCTGA TTATTATATT TACAAGCACT GTATATTGTG 3720  
 20          CTATTCTAAG ATGTGCTAAA CGGATTTGGG GAATGCAACT AACTGCTGTA AGGTATCAAC 3780  
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 25          ATGCTGACAC CCTAGCGGCA TGTTGCGGT ATTGCACTAC AGCGGCAACA ATGGTAAAAA 3960  
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 30          GTAACACTCT GTCAAGGAGA ACATATCACC TTAAGGGTAC ATATAGTAGT TTTCTTCTAA 4140  
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 35          AGAAAGGCCG TAACAGCCGT GATTAAACAA CAAACATTG ATGTTAGAGC GGCTATTAAA 4320  
 GCTTCTGGTC TGAAGCAATA TGAGGTAGCT ACTTTGATGA ATGTTTCAGC TAGCTATCTC 4380  
 AGCCAGCTTT TACTTCAACC ATTGTCAGAA GGCCATAAGA AGCCGATTAT GGCGGCGATT 4440  
 40          AAACAAGGCG AGTCATTGAA GGGAGAACAA GAATAATGAT GAGCTTAGAA GAACGTGAGC 4500  
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 45          GGCTTCAGGA ATTGCTGGCA TTGAACAAGA CCATTAGCGA CGGTCTGAAC GGCATTGAAA 4680  
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5 GAAGGCCTTA CAGGCCGGCT CTGTCAAGCG TGAAGAGGGC TACAACTCAG AGGGCTTAGA 4860  
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 GGCCAGGAAT GGCTGGCTTT TGTTTTGCCT TCAGACGCTC TCAGAACGCTC ATAGAGCCCC 8100  
 TCTGAGCCTG CATTGGTAGA TTTTCCGGC CGAACACCCCC . 8140

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## (3) Information for sequence ID No 2.

## 5 (i) Sequence characteristics:

- (A) Length: 1202 base pairs
- (B) Type: Nucleic acid
- (C) Strandedness: Double
- (D) Topology: Linear

## 10 (ii) Molecule type: DNA (synthetic).

## (xi) Feature:

(vi) Original source: Lactobacillus bulgaricus

- (A) Name/key: lacS promotor
- (B) Location: 1..239

## (ix) Feature:

(vi) Original source: Staphylococcus aureus

- (A) Name/key: Chloramphenicol acetyltransferase peptide
- (B) Location: 240..890

## (ix) Feature:

(vi) Original source: Lactococcus lactis

- (A) Name/key: stem-loop terminator following galT gene
- (B) Location: 903..1102

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GAATTCACCA ACGCTTCAT TTACGCCTC CCGAAGTACA TGCAAGAGGC TATATGCCA 60  
 TCATTAGCAG CTTAATTGAA TATTTACTGG CTAAACTATT GAGTTTCAA GGCTTCATAG 120  
 30 TTCTTTTG G TGTGGAAGTT TAAATTACTA AAAATATTT AGTAAAACAT CTTGGTTAT 180  
 TTAGTAAACA AGTCTATACT GTAATTATAA ACAAGTTAAC ACACCTAAAG GAGAATTCA 240  
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 ACATAAAACA AGAAGGATAT AAATTTTACC CTGCATTTAT TTTCTTAGTG ACAAGGGTGA 420  
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 45 TTTCTATTAT TCCATGGACT TCATTTACTG GGTTTAACCTT AAATATCAAT AATAATAGTA 720  
 ATTACCTTCT ACCCATTATT ACAGCAGGAA AATTCAATTAA TAAAGGTAAT TCAATATATT 780  
 TACCGCTATC TTTACAGGTA CATCATTCTG TTTGTGATGG TTATCATGCA CGATTCTTA 840

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TGAACTCTAT TCAGGAATTG TCAGATAGGC CTAATGACTG GCTTTATAA TATGAGATAA 900  
5 TCGAAAAAAA AAAGCTCAAA TTTTGAGCT TTTTTGTAT GTAATTGTCA TGCATGAAAA 960  
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AAGACAGTTA AGAAGAAATA AAAATAAATT TAAAAGAGTA TCACTAGCTT TTTTTGGTTT 1080  
10 AGTGATTATT TTAGCGGACC TC 1102

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## SEQUENCES LISTING

## 10 (1) GENERAL INFORMATION :

## (i) APPLICANT:

- (A) NAME: SOCIETE DES PRODUITS NESTLE S.A.
- (B) STREET ADDRESS: P.O.Box 353
- (C) CITY: VEVEY
- (D) COUNTRY: SWITZERLAND
- (F) POSTAL CODE: 1800
- (G) TELEPHONE: (21) 924 21 39
- (H) FAX: (21) 921 18 85
- (I) TELEX: 451 311

15 (ii) TITLE OF INVENTION: Plasmid derived from Lactobacillus bulgaricus  
 20 (iii) NUMBER OF SEQUENCES: 6

## (iv) MANDATORY INFORMATIONS:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) Information for SEQ ID NO: 1:

## (i) Sequence characteristics:

- (A) Length: 8140 base pairs
- (B) Type: Nucleic acid
- (C) Strandedness: Double
- (D) Topology: Circular

35 (ii) Molecule type: DNA (plasmid)

(vi) Original source: Lactobacillus bulgaricus Strain N2.  
 (A) Name/key: Plasmid pN42  
 (B) Location: 1..8140

40 (ix) feature:  
 (A) Name/Key: Origin of replication.  
 (B) Location: 5694..5758.

(ix) feature:  
 (A) Name/Key: ORF1.  
 (B) Location: 1344..169.

45 (ix) feature:  
 (A) Name/Key: ORF2.  
 (B) Location: 5965..7806.

(ix) feature:  
 (A) Name/Key: ORF3.  
 (B) Location: 4718..5668.

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## (ix) feature:

(A) Name/Key: ORF4.  
 (B) Location: 3116..3637.

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## (ix) feature:

(A) Name/Key: ORF5.  
 (B) Location: 1779..2360.

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CCTAGGCTTG AAATTGACGC ATAGGCGCAA AGGGAGCGGG CGACAGGGGG TAAAGCACGA 60  
 TAAATTCGTT TTTTACAGAC GTTCAGTCCA TGTTGTCATA TTGTACTCC CGTTTTAGG 120  
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 25 TTTTATATTC TTCAATGTAT TAAACATAAA ATAGAAATTG TCAGAATTAT CATTCAAAC 420  
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CAGACTGACT ATCTTCAGAA GCGGGGAATC ACTCAGACAA CAGCAGAGAT TTTCAATTAA 6480  
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 CAGAGGGACT TAGAAGCCCT GGGCTTACT TGCTATGTCA ACCCGGTTAA CGGCGACTAC 6900  
 20 AAGGACGCTA ACGAGTTCCCT GGTAAGGAT AGAGAGGGCT TCAGACAGAA ACTTCAGCAC 6960  
 GTCATCAATC AGCCCGACAA TTGGCTTGAC AATTACTATG CTGACATCAA AAAACGCCAT 7020  
 GACTACCCGG ACAATATCCC TACTGGCTTC AAGAATTAG ATGATGAGCT TGACGGCGGT 7080  
 25 CTTCAAGCCTA AACTGTATGT TTTAGGCGCT GTCAAGTCGC TAGGGAAAAC GACTTTGCC 7140  
 TTGAATATTG CTGACAACCT GGCTAACAG GGGAGACATG TTTTCTTCTT CAGCATGGAA 7200  
 TCTAGCAAGA GAGAAGTGAC GGACAAGCTT TTAAGCCGGG CTAGCTGTCT CTCTAACGGC 7260  
 30 CATAAAATGGA CTCAGCTTCA AGTCAGCCGG GGAGAATGGT TGAACAATGC TGAGGACAAA 7320  
 GAAGAGTTG ACGGCCTGTT TAAAGCCTTC AGCCGTTACC AGCACTTCTT ACATATCTAT 7380  
 GACAATAGAG TTAAGGCAAG TCAGGTAAAA GACCTGGTCA ATAGTTGGCT TGACAACCCAC 7440  
 35 CCGGACCGAGA AGAAGCCGCT TGTAGTCGTT GACTATCTTC AGATCTTGCA AGCTGAGCAG 7500  
 GACAATGTGA CAGATAAGGC GAAAGTGACG GACAGCGTGA GTGTTCTCTC AGAGCTGACT 7560  
 AAACAGGCTG AAGTCCCTGT TCTGGTCATC TCATCATTGA ACCGGGCTTC CTACTGGCAA 7620  
 40 GACGTAAGTT TTGAATCCTT CAAGGAATCC GGGGAAATTG AGTACTCAGC AGACGTTATG 7680  
 TTAGGATTAG AGTTCGCTCA TCGTGAAGAA TACATTACAG TTAAGGGCAA CGGCCATGTT 7740  
 GAATTGAACA AAGAGAAGTT TGACCAGCGG AAACAGGAAG TCCTAGACGG GTTGAAATGG 7800  
 45 TCATTCTGAA GAATCGAACT GGCAAGACAG GCGGTCAATAT CTTCTTCAAG TACAACGCCA 7860  
 TGTTTAACAG CTACCAGGCA TGCACGTGAGC AAGAGGCCGC AATACCCAAT AACTTTAATA 7920  
 AGTTGTTCA TAGCAAGGAA GTAGGCAAGC CAATTGAAGC GGCTGTGCGT GATTACACGG 7980  
 50 TAGACCCGGT AACAGGCCTG GCAACAGAGA AGAAGCCCGA TAAATAGAAC TGAAGAAGCT 8040  
 GGCCAGGAAT GGCTGGCTT TGTTTGCCCT TCAGACGCTC TCAGAAGCTC ATAGAGCCCC 8100

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TCTGAGCCTG CATTGGTAGA TTTTCGGC CGAACACCCC

8140

## 10 (3) Information for SEQ ID NO: 2:

## (i) Sequence characteristics:

- (A) Length: 1202 base pairs
- (B) Type: Nucleic acid
- (C) Strandedness: Double
- (D) Topology: Linear

## (ii) Molecule type: DNA (synthetic)

20 (vi) Original source: Lactobacillus bulgaricus  
 (A) Name/key: lacS\_promotor  
 (B) Location: 1..239(vi) Original source: Staphylococcus aureus  
 (A) Name/key: Chloramphenicol acetyltransferase peptide  
 (B) Location: 240..89025 (vi) Original source: Lactococcus lactis  
 (A) Name/key: stem-loop terminator following galt gene  
 (B) Location: 903..1102

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCACCA ACGCTTCAT TTACGCCTC CCGAAGTACA TGCAAGAGGC TATATGCCA 60  
 TCATTAGCAG CTTAATTGAA TATTTACTGG CTAAACTATT GAGTTTCAA GGCTTCATAG 120  
 TTCTTTTGG TGTGGAAGTT TAAATTACTA AAAATATTTT AGTAAAACAT CTTGGTTTAT 180  
 TTAGTAAACA AGTCTATACT GTAATTATAA ACAAGTTAAC ACACCTAAAG GAGAATTCA 240  
 TGAACCTTAA TAAAATTGAT TTAGACAATT GGAAGAGAAA AGAGATATT AATCATTATT 300  
 TGAACCAACA AACGACTTTT AGTATAACCA CAGAAATTGA TATTAGTGTGTT TTATACCGAA 360  
 ACATAAAACA AGAAGGATAT AAATTTTACC CTGCATTAT TTTCTTAGTG ACAAGGGTGA 420  
 TAAACTCAA TACAGCTTTT AGAACTGGTT ACAATAGCGA CGGAGAGTTA GGTTATTGGG 480  
 ATAAGTTAGA GCCACTTTAT ACAATTGGT ATGGTGTATC TAAAACATTG TCTGGTATT 540  
 GGACTCCTGT AAAGAATGAC TTCAAAGAGT TTTATGATT ATACCTTCT GATGTAGAGA 600  
 AATATAATGG TTCGGGGAAA TTGTTCCCA AAACACCTAT ACCTGAAAAT GCTTTTCTC 660  
 TTTCTATTAT TCCATGGACT TCATTTACTG GGTTAACCTT AAATATCAAT AATAATAGTA 720  
 ATTACCTTCT ACCCATTATT ACAGCAGGAA AATTCAATTAA TAAAGGTAAT TCAATATATT 780  
 TACCGCTATC TTTACAGGTA CATCATTCTG TTTGTGATGG TTATCATGCA GGATTGTTA 840

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TGAACTCTAT TCAGGAATTG TCAGATAGGC CTAATGACTG GCTTTATAA TATGAGATAA 900  
 TCGAAAAAAA AAAGCTAAA TTTTGAGCT TTTTTGTAT GTAATTGTCA TGCATGAAAA 960  
 10 TGTAATGGTA ATTGTGATAA TTATTAATAA AAAAATTGAT ATAATGAAGT GGATGAAAAA 1020  
 AAGACAGTTA AGAAGAAAATA AAAATAAATT TAAAAGAGTA TCACTAGCTT TTTTTGGTTT 1080  
 AGTGATTATT TTAGCGGAGC TC 1102

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20 (4) Information for SEQ ID NO: 3:

(i) Sequence characteristics:

- (A) Length: 33 base pairs
- (B) Type: Nucleic acid
- (C) Strandedness: Single
- (D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

30 AGGAGGATCC TCTCATGAAC TTTAATAAAA TTG

33

35 (5) Information for SEQ ID NO: 4:

(i) Sequence characteristics:

- (A) Length: 26 base pairs
- (B) Type: Nucleic acid
- (C) Strandedness: Single
- (D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

45 TACAGTATCG ATTATCTCAT ATTATA

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## (6) Information for SEQ ID NO: 5:

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## (i) Sequence characteristics:

10 (A) Length: 31 base pairs  
 (B) Type: Nucleic acid  
 (C) Strandedness: Single  
 (D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

15 ATTGGAAAGAA TTCACCAACG CTTTCATTTC

31

## 20 (7) Information for SEQ ID NO: 6:

## (i) Sequence characteristics:

25 (A) Length: 19 base pairs  
 (B) Type: Nucleic acid  
 (C) Strandedness: Single  
 (D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

30 GGTGGTGACG AAGACGATA

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**Claims**

1. Plasmid derived from *Lactobacillus delbrueckii* sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof.
2. Plasmid according to claim 1, characterized in that the portion is a sufficient amount of the restriction map of the Figure 1, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in *Lactobacillus bulgaricus*.
3. Plasmid according to claim 1 or 2 comprising at least the DNA sequence SEQ ID N° 1 and/or its complementary strand or portion(s) thereof.
4. Plasmid according to claim 3, characterized in that the portion is a sufficient amount of the DNA sequence SEQ ID N° 1, and/or its complementary strand, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in *Lactobacillus bulgaricus*.
5. Recombinant vector comprising the plasmid according to any of the preceding claims, at least one DNA sequence capable of replication in *E. coli* and/or *Lc. lactis* and at least one marker.
6. Microorganism transformed by the plasmid according to any of the claims 1 to 4 and/or by the recombinant vector according to claim 5.
7. *Lactobacillus bulgaricus* transformed by the plasmid according to any of the claims 1 to 4 and/or by the

recombinant vector according to claim 5.

8. Use of the plasmid according to any of the claims 1 to 4 and/or the vector according to claim 5 for the transformation of microorganisms.

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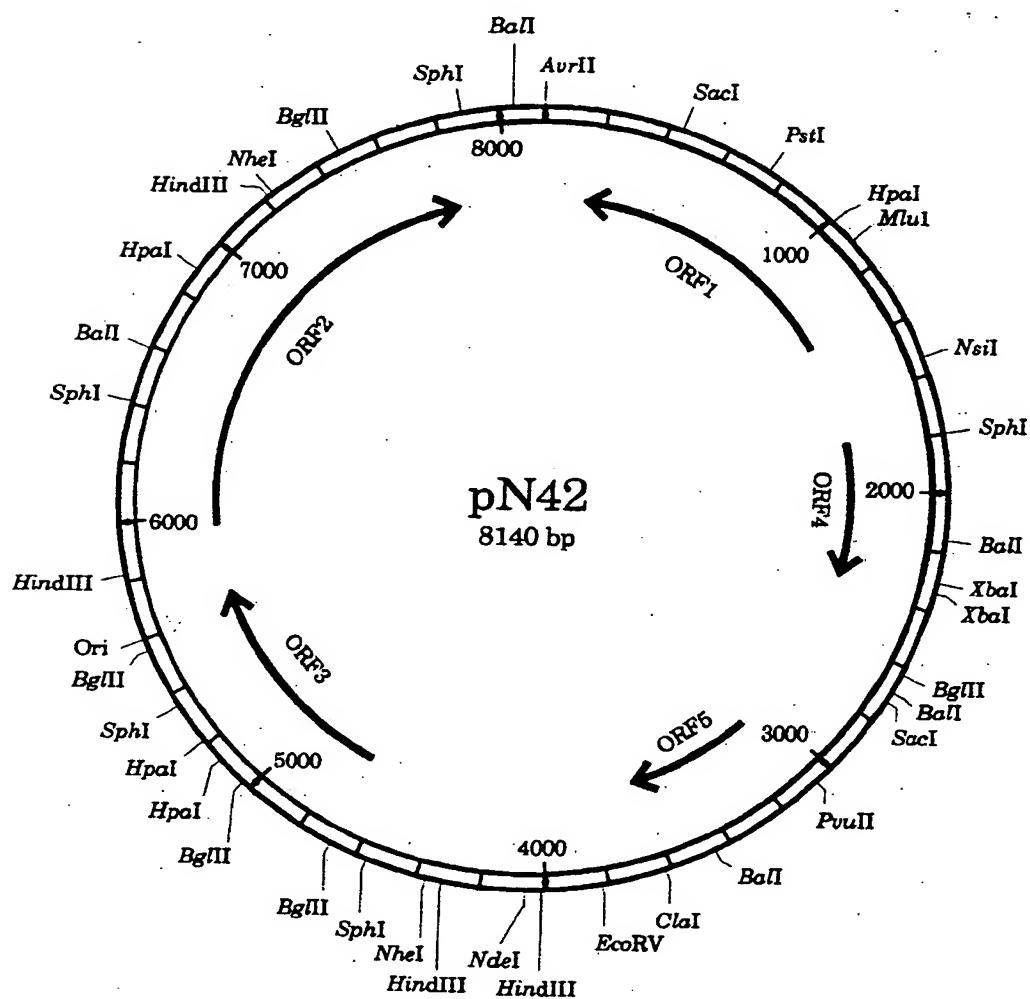
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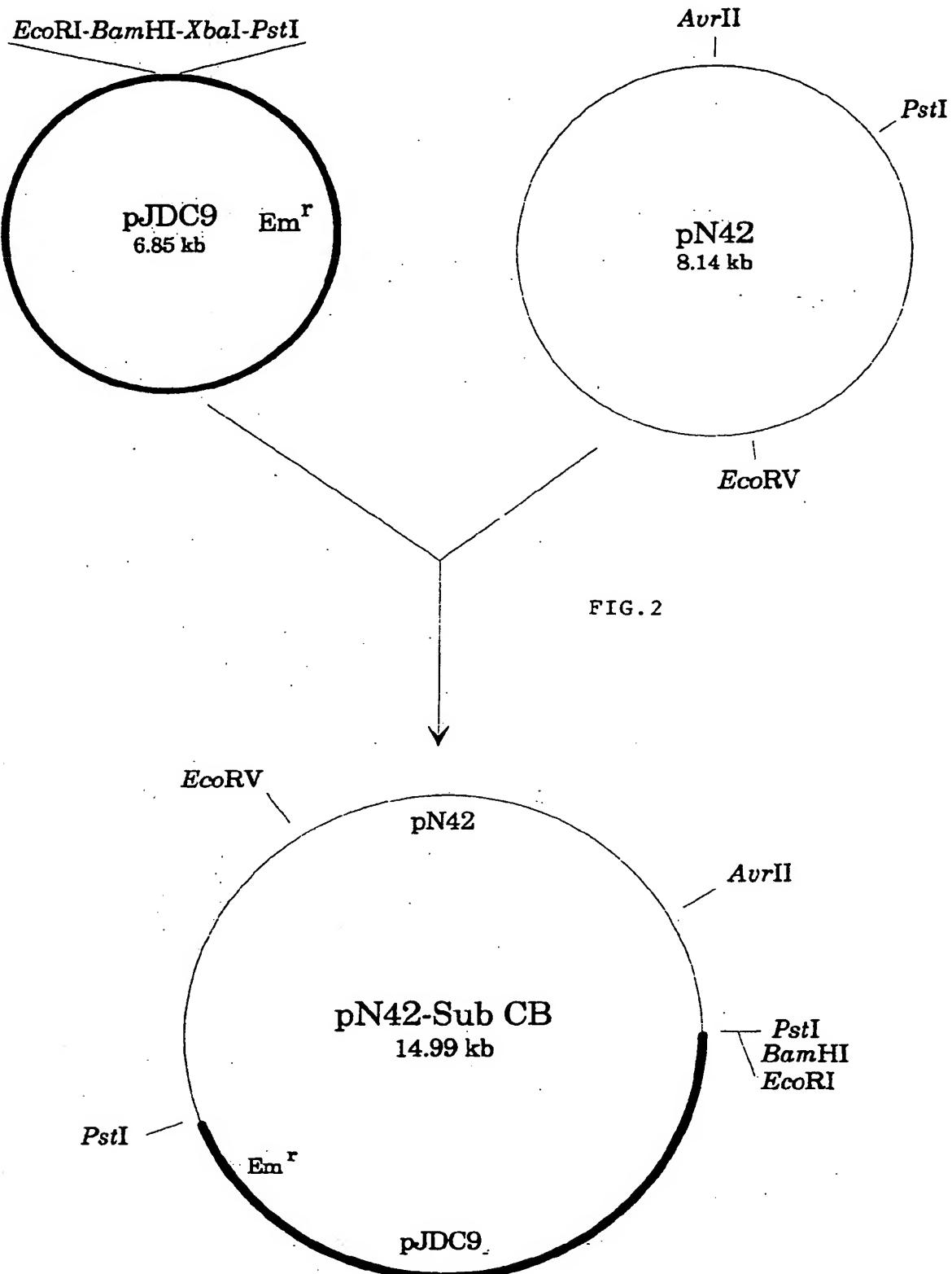
45

50

55

FIG. 1





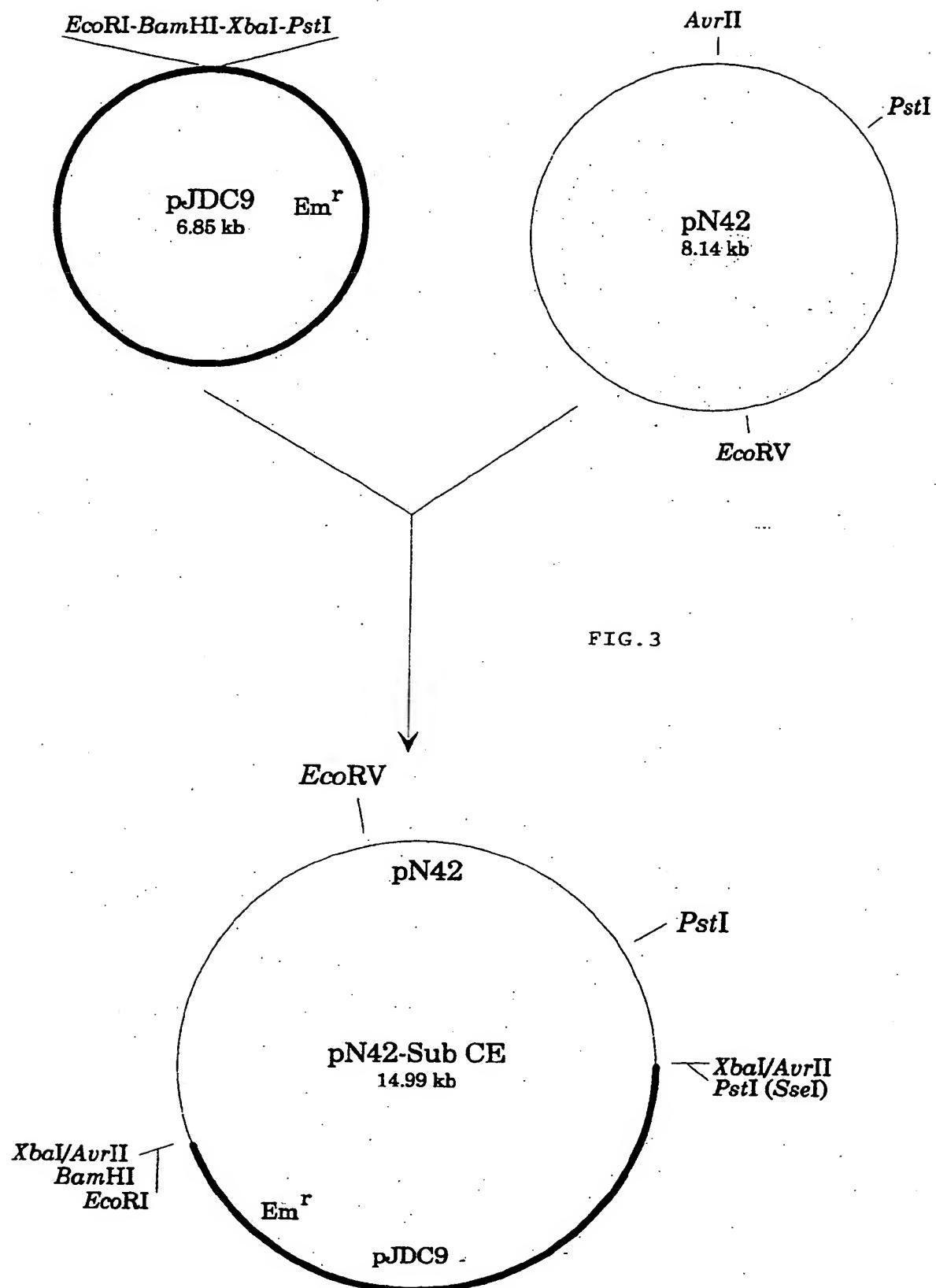


FIG. 3

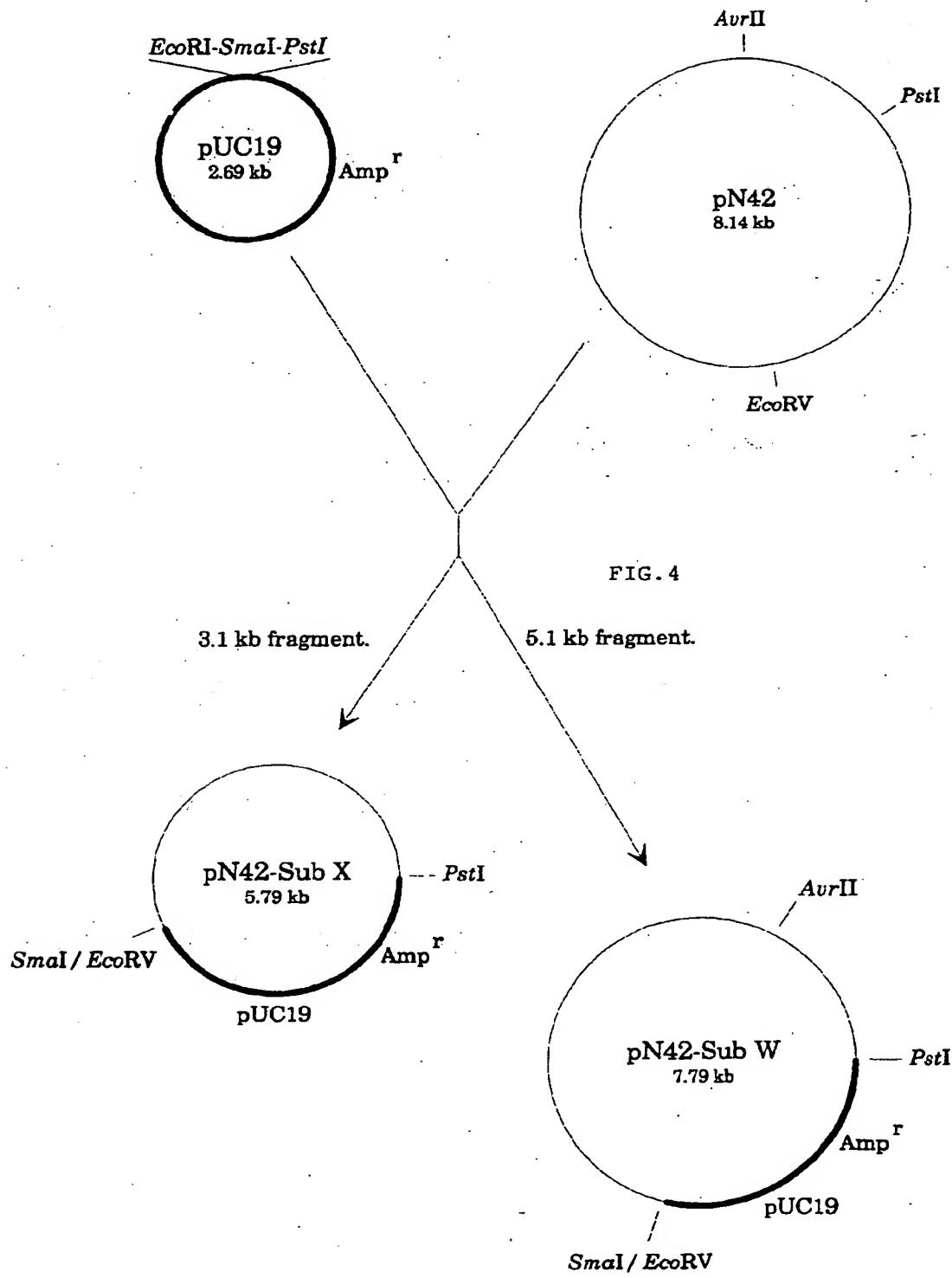
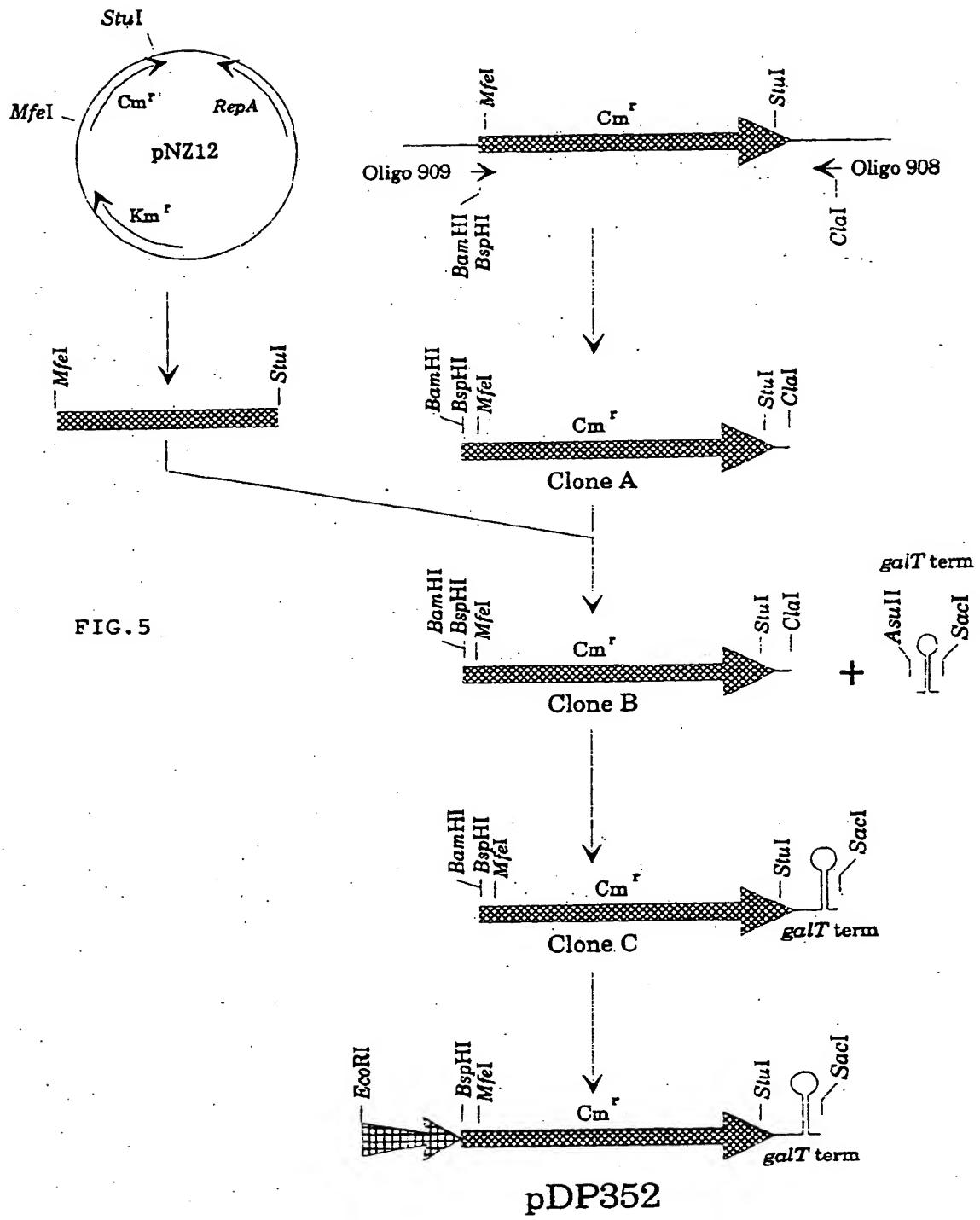
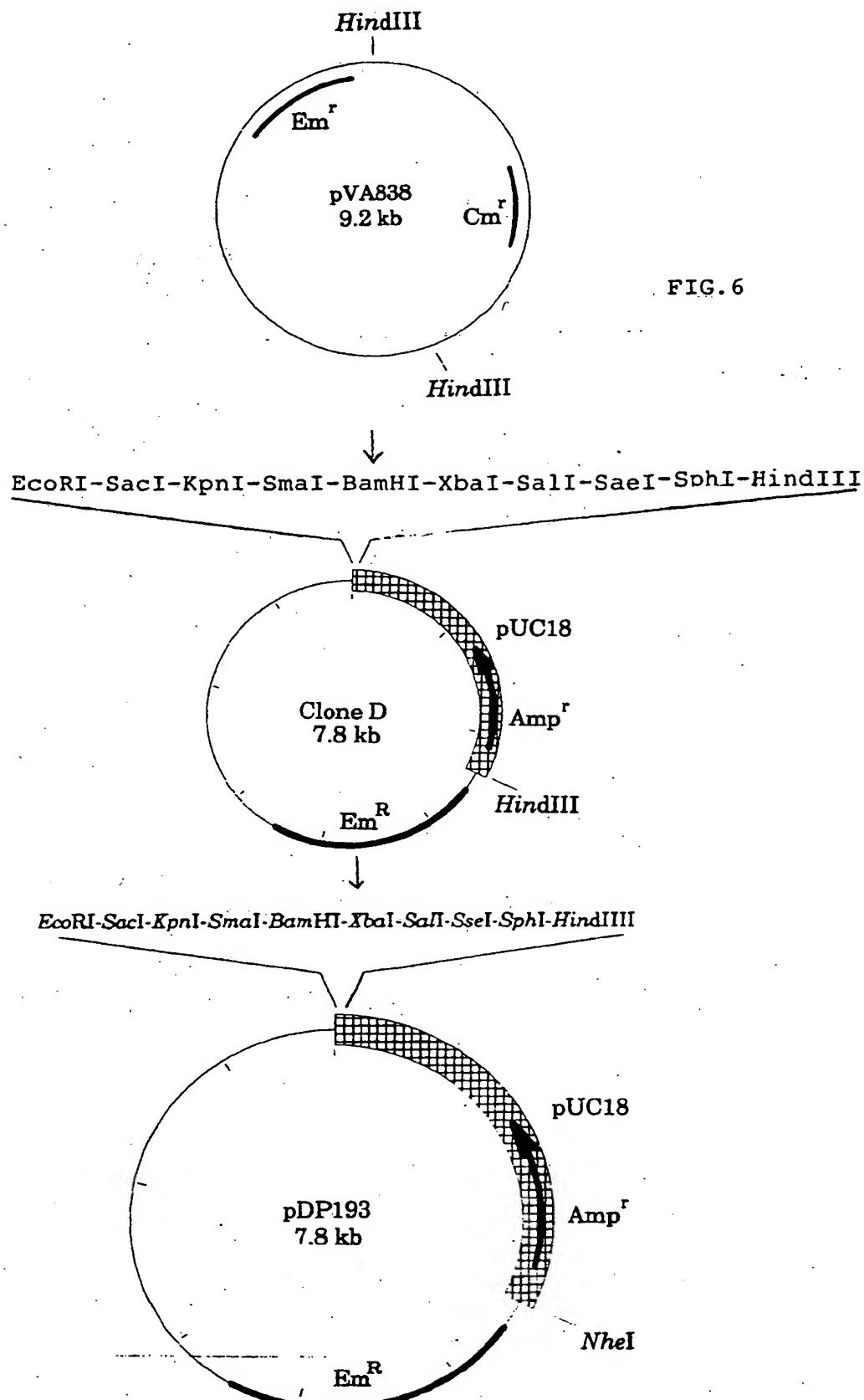


FIG. 4





EcoRI-SacI-KpnI-SmaI-BamHI-XbaI-SalI-SseI-SphI-HindIII

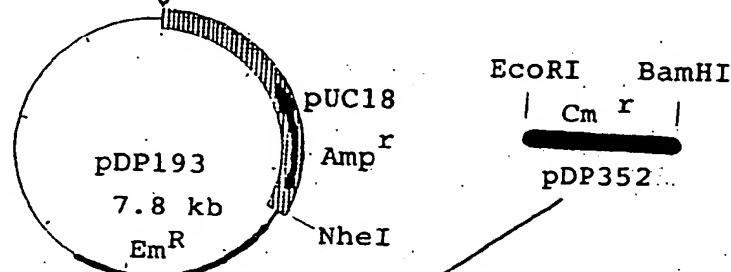
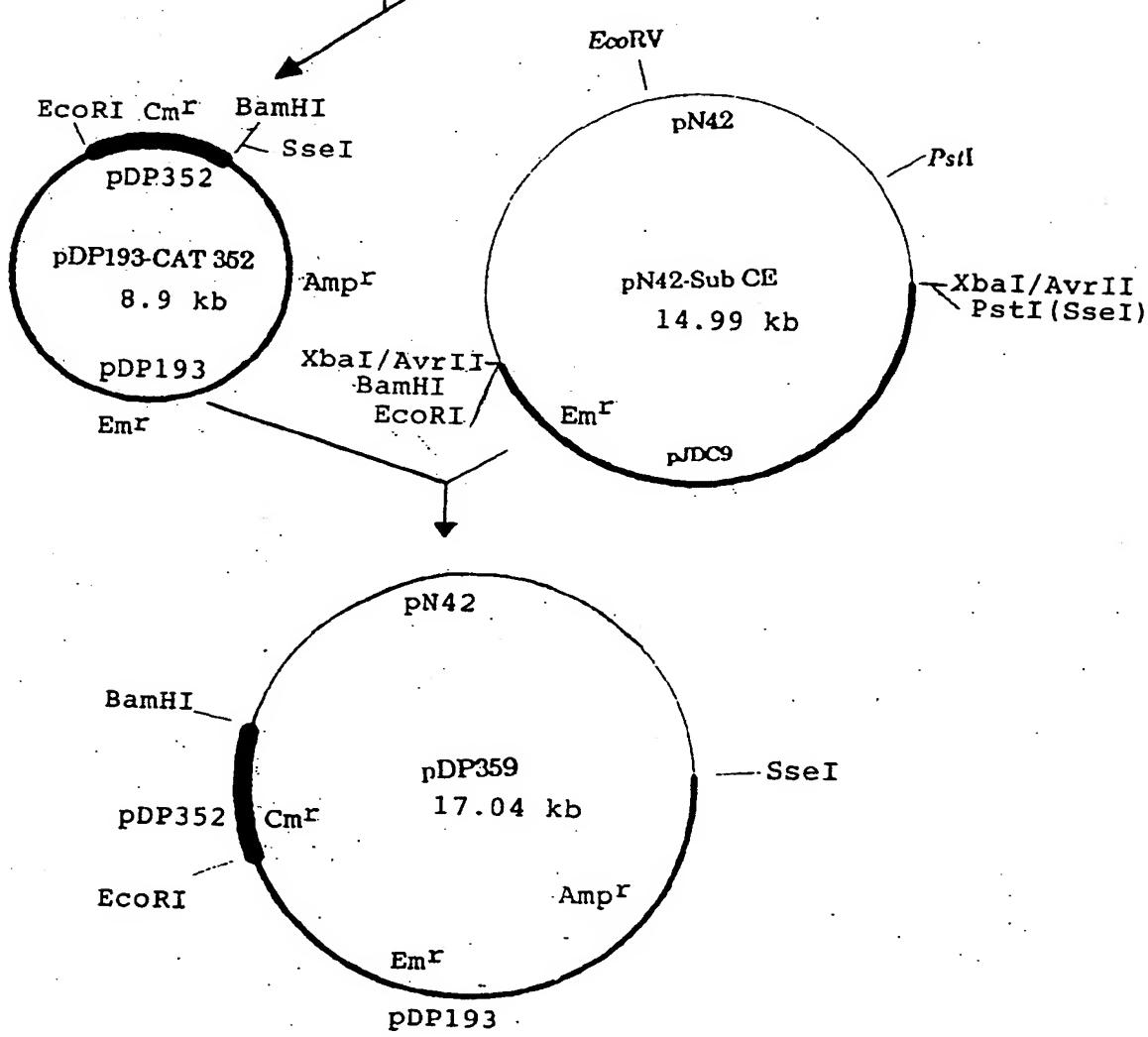


FIG. 7





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## EUROPEAN SEARCH REPORT

Application Number  
EP 94 20 2468

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	EP-A-0 529 088 (MEIJI MILK PROD. CO., LTD.) 3 March 1993 * the whole document * ---	1-8	C12N15/74 C12N1/21 //(C12N1/21, C12R1:225)
A	JAPANESE PATENTS ABSTRACTS (UNEXAMINED) Week 9238, Derwent Publications Ltd., London, GB; AN 92-312519 & JP-A-4 218 381 (SNOW BRAND MILK PROD CO LTD) 7 August 1992 * abstract *	1-8	
A	CAN. JOURNAL OF MICROBIOLOGY, vol.38, 1992, NATL. RESEARCH COUNCIL, OTTAWA, CAN; pages 69 - 74 P. CHAGNAUD ET AL. 'Construction of a new shuttle vector for Lactobacillus' * the whole document *	1-8	
A	ACTA MICROBIOLOGICA BULGARICA, vol.27, no.0, 1991, BULGARIAN ACADEMY OF SCIENCES, SOFIA, BULGARIAN; pages 3 - 8 V. MITEVA ET AL. 'Isolation and characterization of plasmids from different strains of Lactobacillus bulgaricus, Lactobacillus helveticus and Streptococcus thermophilus' * the whole document *	1-8	TECHNICAL FIELDS SEARCHED (Int.Cl.6)  C12N
D,A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol.56, no.6, June 1990, AM. SOC. MICROBIOL., WASHINGTON, DC, US; pages 1967 - 1970 M. DELLEY ET AL. 'DNA probe for Lactobacillus delbrueckii' * the whole document *	1-8 -/-	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	16 December 1994	Hornig, H	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
O : non-written disclosure	L : document cited for other reasons		
P : intermediate document	& : member of the same patent family, corresponding document		



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 94 20 2468

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,A	<p>JOURNAL OF BACTERIOLOGY, vol.173, no.6, March 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 1951 - 1957</p> <p>P. LEONG-MORGENTHALER ET AL. 'Lactose metabolism in Lactobacillus bulgaricus: Analysis of the primary structure and expression of the genes involved' * the whole document *</p> <p>-----</p>	1-8	
			TECHNICAL FIELDS SEARCHED (Int.Cl.)
<p>The present search report has been drawn up for all claims</p>			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	16 December 1994	Hornig, H	
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention      E : earlier patent document, but published on, or          after the filing date      D : document cited in the application      L : document cited for other reasons      &amp; : member of the same patent family, corresponding          document</p>	
<p>X : particularly relevant if taken alone      Y : particularly relevant if combined with another          document of the same category      A : technological background      O : non-written disclosure      P : intermediate document</p>			